

Cyclic AMP-dependent Mechanism Regulates Acetylcholine Receptor Function on Bovine Adrenal Chromaffin Cells and Discriminates between New and Old Receptors

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Abstract. Bovine adrenal chromaffin cells have nicotinic acetylcholine receptors (AChRs) that mediate release of catecholamines from the cells in response to synaptic input, and resemble neuronal AChRs in pharmacology and antigenic profile. Results presented here show that a cAMP-dependent process enhances the function of adrenal chromaffin AChRs as a population in the plasma membrane. This was demonstrated by showing that cAMP analogues cause specific increases both in the level of nicotine-induced catecholamine release from the cells and in the level of the nicotine-induced conductance change occurring in the cells. Neither de novo synthesis of receptors nor transport of

preexisting intracellular receptors to the plasma membrane is necessary for the enhancement. The responsiveness of AChRs to regulation by the cAMP-dependent process appears to depend on the length of time the receptors have been on the cell surface. AChRs newly inserted into the plasma membrane generate a greater nicotinic response than do older AChRs and, unlike older AChRs, their response to agonist is not enhanced after treatment of the cells with cAMP analogues. The findings indicate that the AChRs and/or associated components undergo a maturation in the plasma membrane that alters their function and their regulation by secondary messenger systems.

NEUROTRANSMITTER receptors in the postsynaptic membrane mediate synaptic transmission at all chemical synapses. Because of this central role, the receptors represent a likely site at which regulatory mechanisms might act to modulate synaptic transmission. Recent studies with nicotinic acetylcholine receptors (AChRs)¹ on chick ciliary ganglion neurons have indicated that a cAMP-dependent mechanism enhances the acetylcholine (ACh) sensitivity of the cells (Margiotta et al., 1987a). The enhancement appears to represent an increase in the number of functional AChRs on the neurons since no changes that could account for the effect are observed in AChR single channel properties. The increased ACh sensitivity, however, occurs in the absence of protein synthesis, implying that it represents a conversion of preexisting nonfunctional AChRs to functional ones (Margiotta et al., 1987a). The source of the nonfunctional AChRs has not been determined. It could derive either from a substantial population of nonfunctional AChRs thought to be present in the plasma membrane (Margiotta et al., 1987b) or from a large population of intracellu-

lar AChRs known to be contained in the neurons (Stollberg and Berg, 1987).

The possibility that the number of functional AChRs on cells can be increased by a cAMP-dependent process suggests ways in which cell-cell interactions might regulate synaptic signaling. Regulation of this type apparently does not apply to AChRs in vertebrate skeletal muscle or electric organ. Activation of cAMP-dependent processes in muscle reduces ACh sensitivity in a manner consistent with the receptors becoming more susceptible to agonist-induced desensitization (Albuquerque et al., 1986; Middleton et al., 1986). Reconstitution experiments have directly demonstrated that cAMP-dependent phosphorylation of electric organ AChRs enhances agonist-induced desensitization of the receptors (Huganir et al., 1986). Neuronal AChRs, however, are known to differ from muscle AChRs in certain pharmacological, structural, and regulatory aspects (Boulter et al., 1986, 1987; Jacob and Berg, 1987, 1988; Patrick and Stallcup, 1977a, b; Whiting and Lindstrom, 1986, 1987). The ability to recruit functional AChRs from a nonfunctional receptor pool by a cAMP-dependent process could provide neurons a useful flexibility in detecting synaptic signals that would be unnecessary at the neuromuscular junction.

AChRs on bovine adrenal chromaffin cells appear to be of the neuronal type. They resemble neuronal AChRs in single

1. *Abbreviations used in this paper:* ACh, acetylcholine; AChR, nicotinic acetylcholine receptor; α -Bgt, α -bungarotoxin; BrACh, bromoacetylcholine; DTNB, 5,5-dithio-bis(2-nitrobenzoate); 8-Br-cAMP, 8-bromoadenosine 3',5'-cyclic monophosphate; ³H-NE, ³H-norepinephrine; IBMX, 3-isobutyl-1-methylxanthine; n-Bgt, neuronal bungarotoxin.

channel properties, pharmacology, and antigenic determinants (Fenwick et al., 1982a; Higgins and Berg, 1987, 1988a, b). This is expected since adrenal chromaffin cells derive from the neural crest and share many properties with sympathetic neurons. As a result, an examination of the adrenal chromaffin AChR is likely to provide information about receptor regulation that is applicable to neurons as well as endocrine cells. Here we report that cAMP analogues enhance the AChR response of bovine adrenal chromaffin cells, and, as in the case of chick ciliary ganglion neurons, do so without detectably increasing the total number of AChRs on the cell surface. The enhanced response involves an activation or conversion of preexisting AChRs on the cell surface, and cannot be explained either by synthesis of new receptors or by the selective transport of functional receptors to the plasma membrane from an intracellular pool. Unexpectedly, the nicotinic response of AChRs newly inserted into the plasma membrane cannot be enhanced by cAMP analogues; only older AChRs are responsive to the treatment. The findings complement observations described in the preceding paper suggesting that AChRs newly inserted into the plasma membrane are more efficacious than older receptors (Higgins and Berg, 1988b).

Materials and Methods

Electrophysiology

The ACh sensitivity of bovine adrenal chromaffin cells in culture was determined using intracellular recording techniques as previously described for neurotransmitter sensitivities of chick ciliary ganglion neurons (McEachern et al., 1985; Smith et al., 1983). In brief, cultures were maintained at 37°C on the stage of a Leitz inverted microscope with phase contrast optics and were continuously perfused with recording medium composed of 137 mM NaCl, 5.4 mM KCl, 0.81 mM MgSO₄, 0.92 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 5.4 mM CaCl₂, 5.6 mM glucose, and 2.5 mM Hepes, pH 7.4. Nicotine was routinely used as agonist rather than ACh so that muscarinic receptors would not be activated. Nicotine at 5 μM was applied to individual cells by pressure (1–3 psi) from a pipette with a 4–6-μm tip positioned 15–30 μm from the cell. Sensitivities measured in this way are likely to represent "summed" responses of most of the functional receptors on the cell since experiments with dye-filled pipettes indicate that the procedure completely immerses the soma in the ejected solution (Choi and Fischbach, 1981; Smith et al., 1983). Chromaffin cells selected for measurement usually had diameters of 25–40 μm and appeared well-attached to, but not flattened against, the substratum. Recordings were made using single intracellular electrodes with resistances of 60–120 megohms in conjunction with an amplifier and Wheatstone bridge circuit (model M701; WPI, Inc., New Haven, CT). Current was injected into a cell to produce a holding potential of –50 mV, unless the resting potential was even more negative. This eliminated contributions from voltage-dependent ionic conductances since the current-voltage relationship was linear up to values of ~–20 mV for the membrane potential, in agreement with previous reports (Fenwick et al., 1982b). Membrane depolarizations in response to nicotine were more negative than –20 mV, and the current-voltage relationship was not changed by 8-Br-cAMP treatment over the range of membrane potentials in which nicotinic responses were recorded. Constant current pulses of 0.08–0.15 nA amplitude and 50 ms duration were delivered. The nicotine-induced membrane conductance (g_{nic}) was calculated as the difference between the maximum input conductance in the presence of nicotine and the resting input conductance immediately before application of the drug. Bath application of d-tubocurarine at 10 μM completely blocked g_{nic} in 15 out of 15 cells tested, confirming that the response was nicotinic as well as demonstrating the pressure injection used to apply nicotine did not by itself elicit a response. Data were accepted if the cell maintained a resting potential more negative than –35 mV in the absence of a holding potential, and the recording electrode was no more than 3 mV out of balance after withdrawal from the cell.

Other Procedures

All other procedures, including preparation of cultures, determination of ¹²⁵I-mAb 35 binding, measurement of ³H-norepinephrine (³H-NE) release, and affinity alkylation of AChRs were carried out as described in the accompanying paper (Higgins and Berg, 1988b).

Materials

8-bromoadenosine 3',5'-cyclicmonophosphate (8-Br-cAMP), 8-bromoguanosine 3',5'-cyclicmonophosphate, dibutyryladenosine 3',5'-cyclicmonophosphate, dibutyrylguanosine 3',5'-cyclicmonophosphate and 3-isobutyl-1-methylxanthine (IBMX) were obtained from Sigma Chemical Co. (St. Louis, MO). All other materials were obtained from the sources indicated in the accompanying paper (Higgins and Berg, 1988b).

Results

Enhancement of AChR Function by cAMP Analogues

Possible regulation of bovine adrenal chromaffin AChRs by cAMP was examined first by incubating adrenal chromaffin cells in culture with membrane permeant analogues of cAMP and measuring nicotine-induced ³H-NE release from the cells as an assay for AChR function. Nicotine was used

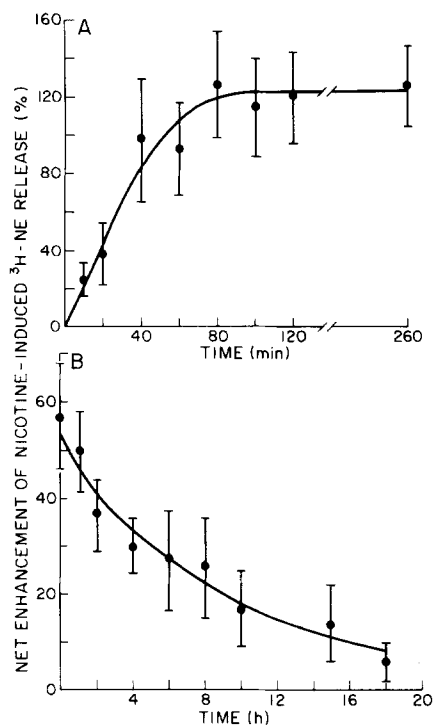


Figure 1. Time course and reversibility of cAMP-dependent enhancement of nicotine-induced ³H-NE release. (A) Adrenal chromaffin cultures were incubated with 0.5 mM 8-Br-cAMP for the indicated times before being rinsed and tested for nicotine-induced ³H-NE release. (B) Cultures were incubated with 0.5 mM 8-Br-cAMP for 12–18 h, rinsed, incubated in culture medium without the cyclic nucleotide for the indicated times, and then tested for nicotine-induced ³H-NE release. Data are expressed as the percent net increase of release over values obtained for untreated cultures and represent the mean ± SEM of three experiments, each performed with triplicate sets of cultures. Lines were fit by eye in A and by linear regression analysis in B.

Table I. Effects of Cyclic Nucleotides and Related Compounds on the Function and Number of AChRs

Treatment	Percent net increase		
	Nicotine-induced ³ H-NE release	K ⁺ -induced ³ H-NE release	¹²⁵ I-mAb 35 binding sites
8 Br-cAMP	66 ± 12* (14)	-9 ± 3 (5)	0 ± 2 (4)
dibutyryladenosine 3',5'-cyclicmonophosphate	51 ± 7* (6)	4 ± 8 (5)	0 ± 12 (4)
8-bromoguanosine 3',5'-cyclicmonophosphate	-9 ± 2 (5)	-10 ± 5 (5)	4 ± 11 (4)
dibutyrylguanosine 3',5'-cyclicmonophosphate	-4 ± 6 (5)	1 ± 7 (5)	6 ± 8 (4)
IBMX	36 ± 3* (4)	1 ± 7 (5)	-2 ± 2 (3)
IBMX + 8-Br-cAMP	75 ± 7* (5)	-2 ± 3 (5)	1 ± 3 (4)
adenosine	-20 ± 9 (6)	ND	17 ± 2 (4)
2-Cl-adenosine	-3 ± 13 (5)	ND	0 ± 3 (3)
AMP	-1 ± 2 (5)	ND	-5 ± 4 (4)

Cultures were incubated with the indicated compound(s) at 0.5 mM for 24 h (except IBMX which was supplied at 1 mM), rinsed, and then tested for ³H-NE release induced by either 1 μM nicotine or 54 mM K⁺, or tested for ¹²⁵I-mAb 35 binding. Basal release obtained in the absence of secretagogue was subtracted in the ³H-NE release assays. Data are expressed as the percent net increase over levels obtained in untreated sister cultures and represent the mean ± SEM for the number of experiments indicated in parenthesis, with each experiment employing triplicate sets of cultures. In control cultures nicotine-induced ³H-NE release was 5.9 ± 1.0% (14) of the total ³H-NE present in the cells at the beginning of the 3-min test period, while K⁺-induced ³H-NE release was 5.8 ± 0.8% (5) of the total in the cells.

* Significantly different from untreated sister cultures, *p* < .005.

routinely as the AChR agonist rather than ACh so that muscarinic receptors would not be activated. Exposure of the cultures to 0.5 mM 8-Br-cAMP at 37°C produced a substantial increase in the amount of nicotine-induced ³H-NE release. A maximum was obtained within 80 min and ranged between 30 and 120% net enhancement among experiments (Fig. 1 A). Because of the variation, results were always compared to controls run in the same experiment, and the ratios averaged among experiments. The enhancement was slowly reversible, having a half-time for decline of ~10 h after the 8-Br-cAMP was removed from the extracellular medium (Fig. 1 B). A second cAMP analogue as well as the membrane permeant phosphodiesterase inhibitor IBMX had a similar effect on the cells (Table I). Combining IBMX and 8-Br-cAMP did not significantly increase the amount of nicotine-induced ³H-NE release over that caused by 8-Br-cAMP alone (Table I). The effect appeared to be specific for cAMP since it could not be mimicked by cGMP analogues or by noncyclic adenine compounds. None of the compounds tested had any effect on the amount of ³H-NE released in response to depolarization of the cells by 54 mM K⁺. The results are consistent with a cAMP-dependent process enhancing the nicotinic response of the cells.

The possibility that cAMP analogues caused an increase in the number of AChRs on the cells was tested by measuring the number of ¹²⁵I-mAb 35 binding sites. mAb 35 has been shown to cross react with the bovine adrenal chromaffin AChR (Higgins and Berg, 1987). No difference in the number of antibody binding sites was detected on the surface of the cells after treatment with any of the compounds (Table I).

To confirm that the cAMP analogues increased AChR function as suggested by the ³H-NE release assay, intracellular recording was used to examine the sensitivity of the cells to nicotine. Untreated control cells displayed a mean *g_{nic}* of 37 ± 6 nS (*n* = 32) in response to a standard test application of 5 μM nicotine. Cells that had first been incubated in 0.5 mM 8-Br-cAMP for 12–18 h displayed a mean *g_{nic}* of 98 ± 11 nS (*n* = 39). Examples are shown in Fig. 2. This constitutes a net increase of 165 ± 35% and confirms the

results of the release assay showing that cAMP analogues do enhance the nicotinic response of bovine adrenal chromaffin cells.

The recordings were made in a linear range of the current-voltage relationship for the cells, and 8-Br-cAMP treatment did not change the current-voltage relationship in the range examined (data not shown). Accordingly, the increased *g_{nic}* caused by the cAMP analogue did not include an increased contribution from voltage-dependent ion channels. The observation that a larger increase is obtained in *g_{nic}* than in nicotine-induced ³H-NE release after 8-Br-cAMP treatment is likely to be due to NE release depending on Ca²⁺ entry which is more proportional to changes in membrane voltage than to changes in *g_{nic}*. The membrane depolarization caused by nicotine increased an average of 56 ± 12% (*n* = 39) after 8-Br-cAMP treatment, which is in good agreement with the mean increase of 66 ± 12% (*n* = 14 experiments) in nicotine-induced ³H-NE release caused by 8-Br-cAMP.

Mechanism of the cAMP-dependent Enhancement

The increased nicotinic response caused by cAMP analogues

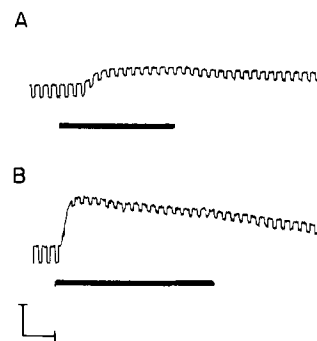


Figure 2. cAMP-dependent enhancement of *g_{nic}*. Adrenal chromaffin cultures were incubated with and without 0.5 mM 8-Br-cAMP for 12–18 h, rinsed, and analyzed with intracellular recording for conductance changes in response to application of 5 μM nicotine. The traces show membrane voltage; downward deflections result from constant current pulses passed through the recording electrode. The duration of nicotine application is indicated by the horizontal bars. (A) Control; *g_{nic}* = 30 nS. (B) 8-Br-cAMP; *g_{nic}* = 85 nS. Vertical bar, 10 mV; horizontal bar, 0.5 s.

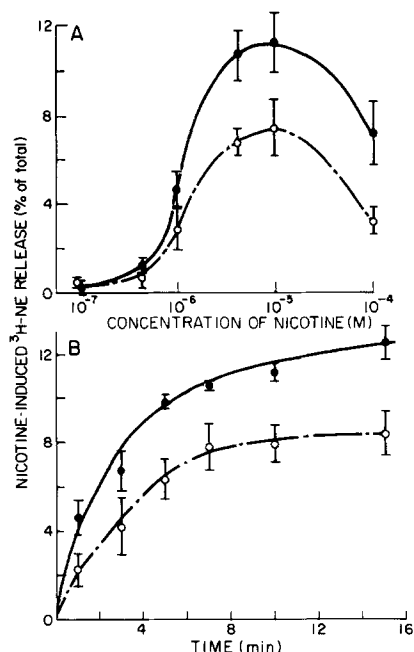


Figure 3. Effects of 8-Br-cAMP on the time course and concentration dependence of nicotine-induced ^3H -NE release. Cultures were incubated with (solid circles) or without (open circles) 0.5 mM 8-Br-cAMP for 12–18 h and then tested for nicotine-induced ^3H -NE release by varying either the concentration of nicotine (A) or the time of exposure to it (B) in the standard assay. Data represent the mean \pm SEM of four experiments in each case, with each experiment involving a triplicate set of cultures.

does not appear to represent a major change either in receptor affinity for agonist or in the kinetics of the secretory response. Both the dependence on agonist concentration and the time course of nicotine-induced ^3H -NE release from the cells were the same after incubation with 8-Br-cAMP (Fig. 3). Only the magnitude of the release was increased by the cyclic nucleotide.

The ^{125}I -mAb 35 binding studies presented above (Table I) suggested that cAMP analogues did not increase the number of AChRs on the cells, and therefore implied that the enhanced nicotinic response must represent an increased efficacy of existing AChRs. This was corroborated by showing that inhibiting synthesis of new receptors using a concentration of puromycin that blocked >98% of protein synthesis did not diminish the ability of 8-Br-cAMP to increase the nicotinic response (data not shown). Accordingly, the increase must represent a change in activity of the existing AChR population.

A possible mechanism for the enhanced nicotinic response is provided by the precedent of cAMP-dependent regulation of AChRs on chick ciliary ganglion neurons. Recent studies suggest that only a fraction of the AChRs on the neurons in culture is functional, and that a cAMP-dependent process can increase the fraction of receptors that are functional (Margiotta et al., 1987a, b). Bovine adrenal chromaffin cells may also have nonfunctional AChR in the plasma membrane. Fenwick et al. (1982a), using patch clamp analysis, estimated that in culture the cells have 100–200 functional AChRs per cell. The number of ^{125}I -mAb 35 binding sites under similar culture conditions is $\sim 1.6 \times 10^4$ per cell

(Higgins and Berg, 1987). Assuming two mAb 35 sites per AChR results in over an order of magnitude discrepancy between the number of AChRs determined with antibody binding and the number determined physiologically. The putative nonfunctional AChRs on the cell surface would contribute a source of receptors that may be converted into a functional form by a cAMP-dependent process. An alternative possibility is that the cAMP-dependent process induces the transfer of preexisting intracellular AChRs to the plasma membrane. About 20% of the total AChRs associated with bovine adrenal chromaffin cells in culture constitute an intracellular pool of receptor of unknown significance (Higgins and Berg, 1988a). If only 5–10% of the surface AChRs are normally functional, and a cAMP-dependent process selectively recruited functional AChRs from the intracellular pool, a two- to threefold increase in nicotinic response might occur without a detectable change in the number of antibody binding sites on the cell surface.

Two types of experiments eliminated the possibility that the cAMP-dependent enhancement of the nicotinic response is mediated predominantly by recruitment of preexisting intracellular AChRs. Both types of experiments employed the strategy of first selectively blocking AChRs on the cell surface and then determining whether cAMP analogues could still induce a nicotinic response. If so, the implication would have been that the cyclic nucleotides recruited AChRs from intracellular pools that had not been inactivated by the treatment with receptor blockers. In the first procedure neuronal bungarotoxin (n-Bgt) was used to block AChRs on the cells (Higgins and Berg, 1987). After washing away unbound n-Bgt and incubating with 8-Br-cAMP, the cells were tested for nicotine-induced ^3H -NE release. It was not practical to use high enough concentrations of n-Bgt to achieve complete block of all previously existing AChRs on the cell surface. Nonetheless, the 8-Br-cAMP treatment increased the amount of agonist-induced release only as much as expected for an effect on the residual surface AChRs remaining unoccupied by n-Bgt; i.e., the amount of nicotine-induced ^3H -NE re-

Table II. Effects of AChR Blockade on Regulation of Nicotinic Response by cAMP Analogues

Treatment	Nicotine-induced ^3H -NE release (% of untreated cultures)		8-Br-cAMP-dependent net enhancement in percent	n
	without 8-Br-cAMP	with 8-Br-cAMP		
None	100	149 \pm 9*	49 \pm 9	(3)
n-Bgt	39 \pm 1	62 \pm 5*	59 \pm 13	(3)
α -Bgt	107 \pm 6	143 \pm 6*	35 \pm 8	(3)
DTT, DTNB	105 \pm 5	163 \pm 9*	55 \pm 10	(7)
DTT, BrACh, DTNB	12 \pm 2	16 \pm 3	ND	(3)
BrACh	98 \pm 9	ND	ND	(3)

Cultures received the indicated treatments and then were rinsed, incubated with or without 0.5 mM 8-Br-cAMP for 2 h, and assayed for nicotine-induced ^3H -NE release. Data on release are presented as a percent of the values obtained from untreated sister cultures, and represent the mean \pm SEM for the number of experiments indicated in parenthesis, with each experiment being done with a triplicate set of cultures. None, no treatment; n-Bgt, 1 μM n-Bgt 1 h before 8-Br-cAMP treatment; α -Bgt, 1 μM α -Bgt 1 h before and 2 h during 8-Br-cAMP treatment; DTT, DTNB, affinity alkylation protocol in which incubation with BrACh was omitted; DTT, BrACh, DTNB, affinity alkylation protocol; BrACh, affinity alkylation protocol with the DTT and DTNB steps omitted. * Significantly different from paired condition without 8-Br-cAMP, $p < .005$.

lease remaining after n-Bgt treatment was increased ~50% by 8-Br-cAMP as was the release in unblocked control cultures (Table II). If the 8-Br-cAMP effect depended on recruitment of intracellular AChRs, the absolute increase in nicotine-induced ³H-NE release brought on by 8-Br-cAMP should not have been diminished by n-Bgt and would have contributed to the secretory response in addition to that resulting from residual unblocked AChRs. The increase would have represented more than a doubling of the residual release observed in the n-Bgt-treated cultures in the absence of 8-Br-cAMP. This was not the case.

In the second procedure, blockade of surface AChRs was achieved by affinity alkylation with bromoacetylcholine (BrACh) as previously described for AChRs from electric organ (Damle et al., 1978) and PC12 cells (Leprince, 1983). Cells were incubated with dithiothreitol (DTT) to reduce sulfhydryls on the cell surface, reacted with BrACh to affinity alkylate AChRs, and reoxidized with DTNB to restore function to nonalkylated AChRs. The procedure specifically blocked the nicotinic response. No loss of K⁺-induced ³H-NE release was observed, and little if any decrement in nicotine-induced ³H-NE release occurred if either the BrACh step or the DTT and DTNB steps were omitted from the protocol. After blockade of surface AChRs by affinity alkylation with BrACh, no increase in the nicotinic response could subsequently be induced by 8-Br-cAMP (Table II). The small amount of nicotine-induced release observed in the experiment arose from new AChRs appearing because of the normal rate of insertion for AChRs in the plasma membrane during the 2-h period between alkylation and assay for nicotine-induced ³H-NE release (Higgins and Berg, 1988b). Both the affinity alkylation experiments and the n-Bgt blockade experiments indicate that the increase in nicotinic response brought on by cAMP analogues is not due to recruitment of intracellular AChRs. This, together with the demonstration that the effect depends on preexisting AChRs, supports the conclusion that the cAMP-dependent enhancement of the nicotinic response involves an effect on existing AChRs in the surface membrane of the cells. This interpretation is in agreement with the observation that 8-Br-cAMP enhancement of the nicotinic response is not accompanied by a detectable increase in the number of ¹²⁵I-mAb 35 binding sites on the cells (Table I).

Bovine adrenal chromaffin cells have α -Bgt receptors that have nicotinic pharmacology, but are distinct from classical AChRs that mediate conductance of monovalent cations (Higgins and Berg, 1987, 1988a). The possibility that cholinergic activation of α -Bgt receptors was involved in the cAMP-dependent effect on AChRs was tested by treating the cells with α -Bgt before and during exposure to 8-Br-cAMP. No effect of α -Bgt was observed on the enhancement of AChR function by the cyclic nucleotide (Table II).

Differences in cAMP-dependent Regulation of New and Old AChRs

The enhancement of the nicotinic response by cAMP analogues described above represents an effect on a population of AChRs with a steady-state distribution of ages in the plasma membrane. Results presented in the accompanying paper suggest that AChR efficacy may vary with receptor age (Higgins and Berg, 1988b). Accordingly, new and old

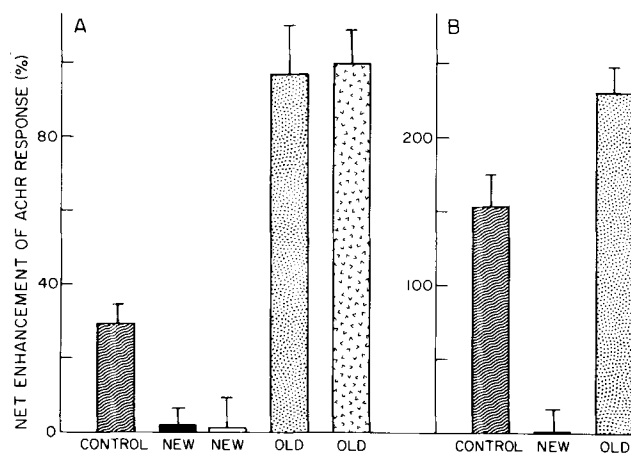


Figure 4. cAMP-dependent enhancement of the nicotinic response from cells with AChRs of different ages. Cells having exclusively new AChRs were obtained either by using antigenic modulation to remove existing AChRs from the cell surface and then allowing the cells to recover for 6 h (solid bars) or by using affinity alkylation with BrACh to block the function of existing AChRs on the cells and then letting them recover for 4 h (open bar). Cells having exclusively old AChRs were obtained by maintaining cells for 27 h in tunicamycin to prevent appearance of new receptors in the plasma membrane (stippled bars). To produce a population of new AChRs that aged in synchrony, cells were allowed to recover from antigenic modulation for 3–4 h and then were maintained in tunicamycin for 24 h before assay (flecked bar). Details of the procedures are provided in the text. The effect of 0.5 mM 8-Br-cAMP for 2 h on either the nicotine-induced ³H-NE release (A) or on the g_{mic} (B) was then determined. Data are expressed as the percent increase in the AChR response caused by 8-Br-cAMP and represent the mean \pm SEM of four experiments performed with triplicate sets of cultures in A and the mean \pm SEM for 24–39 cells for each of the conditions shown in B.

AChRs were examined separately for regulation by 8-Br-cAMP. New AChRs were obtained in isolation by using antigenic modulation with mAb 35 to clear existing AChRs from the cell surface (Higgins and Berg, 1988b). A 6-h recovery period then allowed newly synthesized AChRs to accumulate in the plasma membrane. 8-Br-cAMP had no effect on the nicotinic response of new receptors (Fig. 4 A). In a second strategy to examine the functional properties of new AChRs, affinity alkylation was used to block completely activation of existing AChRs on the cells. After a 4-h recovery period to permit the accumulation of new AChRs on the cells, measurements of nicotine-induced ³H-NE release again demonstrated that 8-Br-cAMP was unable to enhance the response of new AChRs (Fig. 4 A). In contrast, shifting the mean age of the AChR population to higher values revealed a marked enhancement of receptor function by 8-Br-cAMP. This was demonstrated by incubating cultures in tunicamycin for 27 h to block appearance of new AChRs, thereby increasing the average age of the remaining AChRs. Cultures treated in this manner displayed an even greater enhancement of nicotine-induced ³H-NE release by 8-Br-cAMP than did control cultures (Fig. 4 A). Neither antigenic modulation nor 27 h in tunicamycin altered the amount of ³H-NE release induced by 54 mM K⁺, indicating that the effects observed on nicotine-induced release were specific.

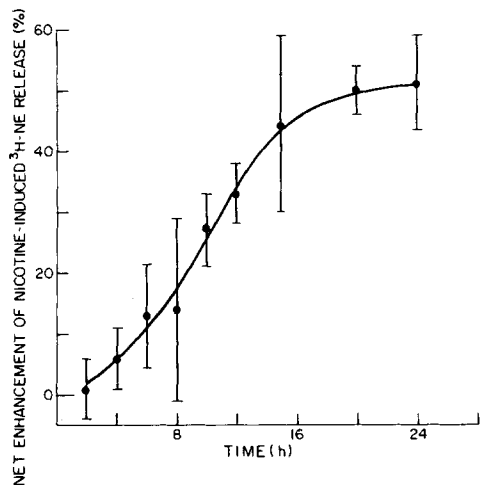


Figure 5. Acquisition of AChR sensitivity to cAMP-dependent regulation. AChRs on the surface of adrenal chromaffin cells in culture were removed by exposing the cultures to mAb 35. The cultures were then allowed to recover for the indicated times before assaying for nicotine-induced ^3H -NE release with and without 0.5 mM 8-Br-cAMP treatment. Cultures receiving 8-Br-cAMP were incubated in the compound starting 4 h before measurement of ^3H -NE release. Data are expressed as the percent enhancement of nicotine-induced ^3H -NE release produced by 8-Br-cAMP, and represent the mean \pm SEM of four experiments done with triplicate sets of cultures.

To show directly that AChRs progress from an 8-Br-cAMP-insensitive stage to a sensitive one, experiments were designed to follow the fate of new AChRs. Cells were treated with mAb 35 to remove surface AChRs and then incubated for 3–4 h to allow new AChRs to accumulate in the plasma membrane. Tunicamycin was then added to the culture medium to prevent additional AChRs from appearing while the existing “new” receptors aged. After a 24-h period in tunicamycin the cells were tested for nicotine-induced ^3H -NE release, with and without a 2-h exposure to 0.5 mM 8-Br-cAMP. The cyclic nucleotide caused a doubling of the nicotine-induced release, showing that AChRs, which are initially insensitive to modulation by the cyclic AMP-dependent process, later became sensitive to it (Fig. 4 A).

Intracellular recording confirmed the results of the release assay, showing differences in the response of new and old AChRs to 8-Br-cAMP treatment. New AChRs were operationally defined in this case as those having accumulated on the cells within 6–8 h after antigenic modulation by mAb 35 to remove preexisting AChRs. Old AChRs were taken to be those present 22–26 h after treatment of the cultures with tunicamycin to prevent the appearance of new AChRs. 8-Br-cAMP treatment had no effect on the nicotinic sensitivity of cells with new AChRs (Fig. 4 B). The compound did enhance the nicotinic response of cells containing old AChRs, and the enhancement was significantly greater ($p < 0.005$) than that caused by 8-Br-cAMP on untreated control neurons having a steady-state distribution of receptor ages.

The rate at which AChRs acquire the ability to be modulated by a cAMP-dependent process was examined by removing surface AChRs through antigenic modulation, allowing the cells to accumulate new AChRs, and then determining the effect of 8-Br-cAMP on nicotine-induced ^3H -NE release

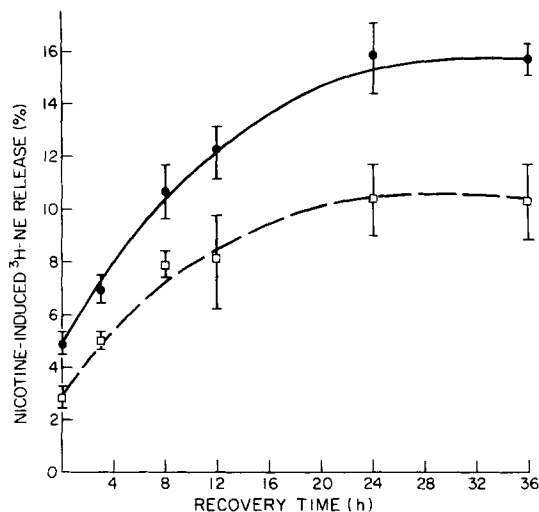


Figure 6. Recovery of AChR sensitivity to cAMP-dependent regulation after partial antigenic modulation of the receptors. Partial removal of AChRs from adrenal chromaffin cells in culture was achieved by exposing the cells to a low concentration of mAb 35. The cultures were then allowed to recover for the indicated times and assayed for nicotine-induced ^3H -NE release. *Solid circles*, 8-Br-cAMP applied at 0.5 mM 4 h before conducting the release assay; *open squares*, cells receiving no cyclic nucleotide. Similar results were obtained in two other experiments.

from the cells at subsequent times. The results demonstrate that within 20 h the cells reach steady-state, having a nicotinic response that is enhanced by 8-Br-cAMP to the same extent as cells with AChRs before antigenic modulation (Fig. 5). The possibility that the time dependence reflected recovery of the cAMP-dependent process itself after antigenic modulation rather than events at the level of AChRs was addressed in the following manner. A partial removal of surface AChRs was achieved by incubating cells with low concentrations of mAb 35. The cells were then exposed to 8-Br-cAMP and tested for nicotine-induced ^3H -NE release. The proportional enhancement caused by the cyclic nucleotide (60%) was identical to that observed with unmodulated sister cultures, indicating that the relevant cAMP-dependent mechanism had not been compromised by the antigenic modulation used to clear AChRs from the cell surface (Fig. 6). The results indicate that a time-dependent maturation of the AChR and/or an associated component confers on the receptor a sensitivity to modulation by a cAMP-dependent process.

Discussion

The results presented here show that a cAMP-dependent process increases the nicotinic response of bovine adrenal chromaffin AChRs, and that it does so by increasing the function of AChRs already present in the plasma membrane. The cAMP-dependent regulation provides, in principle, a mechanism by which cell-cell interactions influencing adenylate cyclase activity could modulate the detection of cholinergic signals by neurons and endocrine cells. Unexpectedly, the effectiveness of the cAMP-dependent process depends on AChR age. AChRs newly inserted into the plasma membrane are not enhanced in function by the process, but later do become responsive to cAMP-dependent modulation.

That a cAMP-dependent process is involved in regulation of AChR function is indicated by the fact that cAMP analogues as well as a phosphodiesterase inhibitor cause an increased nicotinic response from the cells while cGMP analogues and noncyclic adenine compounds have no effect. That the process is AChR-dependent is demonstrated by the finding that nicotine-induced $^3\text{H-NE}$ release is enhanced by cAMP analogues while K^+ -induced $^3\text{H-NE}$ release is not. Intracellular recording confirms the increased AChR-mediated response, showing a substantial increase in g_{nic} for cells incubated with 8-Br-cAMP. The extent of the increase, 165%, is significantly greater than the mean increase of 66% observed for nicotine-induced $^3\text{H-NE}$ release. The difference in results presumably arises from the fact that g_{nic} reflects a change in membrane conductance while $^3\text{H-NE}$ release depends on Ca^{++} entry and therefore on changes in membrane potential needed to activate voltage-dependent calcium channels. Membrane conductance and membrane potential would not be expected to increase in parallel since the driving force on ions changes as a function of voltage. Indeed, the 56% net enhancement of the nicotine-induced membrane depolarization after 8-Br-cAMP treatment agrees well with the 66% net enhancement of the secretory response. Measurement of membrane conductance change provides a more accurate assessment of relative AChR activation than does the release assay. Nevertheless, results from the two kinds of assays are in good qualitative agreement under the conditions used, and lead to the same conclusions about AChR regulation by cAMP.

Conflicting accounts have appeared describing the effects of cAMP analogues on bovine adrenal chromaffin AChRs (Adams and Boarder, 1987; Cheek and Burgoyne, 1987; Marriott et al., 1988; Morita et al., 1987a, b). Generally, agents thought to increase or substitute for cAMP have been reported either to increase nicotine- and K^+ -induced catecholamine release in parallel or to inhibit nicotine-induced release. We have no explanation for the discrepancies other than to point out that different conditions were used to prepare the cells, to activate cAMP-dependent processes, and to assay receptor-mediated catecholamine release. In the present studies intracellular recording unequivocally demonstrated that cAMP analogues did increase AChR function in bovine adrenal chromaffin cells under the conditions used.

Several lines of evidence indicate that the enhanced nicotinic response brought on by cAMP occurs by influencing AChRs already present in the plasma membrane. The increase in nicotine-induced $^3\text{H-NE}$ release caused by 8-Br-cAMP occurs relatively rapidly. The half-time for onset of the increase is ~ 30 min which presumably reflects the time requirement for adequate penetration of the plasma membrane by the cAMP analogue. The increase is not blocked by inhibiting protein synthesis and it is not accompanied by a detectable increase in the total number of AChRs on the surface of the cells. The latter finding suggests that AChRs are not drawn from the population of intracellular receptors to produce the increased response. This conclusion is also supported by the observation that functional blockade of AChRs in the plasma membrane before incubating the cells with 8-Br-cAMP prevents the cyclic nucleotide from enhancing the secretory response. The methods of blockade, i.e., incubation with n-Bgt or affinity alkylation with BrACh, should not have affected intracellular AChRs which would

have been inaccessible to the blocking reagents and therefore still available for transfer to the plasma membrane by 8-Br-cAMP, had this served as the mechanism.

The mechanism of enhancement requires further study. Patch clamp analysis of AChRs on chick ciliary ganglion neurons indicates that cAMP analogues increase the ACh response of the cells by converting nonfunctional AChRs to functional receptors, rather than by changing the single channel properties of functional AChRs (Margiotta et al., 1987b). It is possible that a similar mechanism is involved here, since, as described earlier, the number of functional AChRs reported on bovine adrenal chromaffin cells (Fenwick et al., 1982a) may be substantially smaller than the total number of AChRs detectable in the plasma membrane (Higgins and Berg, 1987, 1988a). Data from the $^3\text{H-NE}$ release assay suggest that the enhanced response caused by 8-Br-cAMP does not involve a change in affinity of agonist for the bovine receptors or in the kinetics of the secretory response to AChR activation. Nonetheless, patch clamp analysis will be required to determine whether a change in single channel properties of the AChR population as a whole might account for the enhanced response. Increases in single channel conductance, mean channel open time, or probability of channel opening, or a decrease in desensitization could cause the observed enhancement and would not be detected by the methods used here. Cholinergic action at α -Bgt receptors (Higgins and Berg, 1988a) does not appear to mediate the cAMP regulation of AChR function since chronic blockade of the receptors with α -Bgt did not prevent 8-Br-cAMP from enhancing nicotine-induced $^3\text{H-NE}$ release.

A reasonable hypothesis for the mechanism by which cAMP analogues enhance AChR function is that they activate a cAMP-dependent protein kinase that phosphorylates either the AChR directly or a related component that acts indirectly to increase the nicotinic response. cAMP-dependent kinases alter the functional state of other types of ion channels (Armstrong and Eckert, 1987; Brum et al., 1983; Ewald et al., 1985; Flockerzi et al., 1986; Shuster et al., 1985). Direct phosphorylation of AChR from electric organ by a cAMP-dependent protein kinase increases agonist-induced desensitization of the receptor (Huganir et al., 1986). A similar regulation may apply to muscle AChRs (Albuquerque et al., 1986; Middleton et al., 1986). AChRs on chick ciliary ganglion neurons display a small increase in agonist-induced desensitization after the cells are incubated with cAMP analogues, but the effect is minor compared to the large increase in the ACh response resulting from the apparent increase in number of functional AChRs that the cAMP analogues cause (Margiotta et al., 1987a). Deduced amino acid sequences for muscle and neuronal AChR subunits suggest differences in the number and location of potential phosphorylation sites for a cAMP-dependent protein kinase (Boulter et al., 1986; Deneris et al., 1988; Goldman et al., 1987; Nef et al., 1988; Wada et al., 1988), and provide a possible molecular basis for the different regulatory effects of cAMP analogues on the two types of AChRs (Margiotta et al., 1987a). A difficulty with the notion that differences in phosphorylation sites account for the different regulatory effects, however, comes from studies on the rat pheochromocytoma cell line PC12. PC12 cells have AChRs that appear to be similar to those of bovine adrenal chromaffin cells (Boulter et al., 1986, 1987) and chick ciliary ganglion neurons (Boyd

et al., 1988) and yet do not show an increased ACh response when challenged with a cAMP analogue or incubated with forskolin to elevate intracellular cAMP levels (Amy and Bennett, 1983; McHugh and McGee, Jr., 1986). The results imply that either PC12 cell AChRs are refractory to the kind of phosphorylation proposed here or that regulation of neuronal AChR function by a cAMP-dependent process involves other events that do not normally occur in the PC12 cell line.

The finding that new and old AChRs on bovine adrenal chromaffin cells differ in their regulation by cAMP suggests that the receptor and/or its associated components exist in different functional states in the membrane. Measurements of both nicotine-induced ³H-NE release and g_{nic} indicate that cAMP analogues are unable to increase the agonist response of AChRs newly inserted in the plasma membrane. Two lines of evidence indicate that the refractoriness of new AChRs in this respect is not a limitation of the regulatory process per se. First, the same refractoriness to cAMP regulation was seen for new AChRs accumulating on cells after antigenic modulation and after affinity alkylation. It is unlikely that both procedures selectively impaired the cAMP-dependent regulatory process in such a manner as to prevent it from acting on new AChRs. Second, when some AChRs were allowed to remain after antigenic modulation, the residual nicotinic response could still be enhanced by cAMP-dependent regulation to the same proportional extent, demonstrating that the regulatory process itself withstood antigenic modulation.

The event that renders AChRs responsive to modulation by a cAMP-dependent process may also be the event that alters the functional state of the receptor as a consequence of age in the plasma membrane. The two changes do occur with similar time courses. One possibility is that the event initially involves a dephosphorylation of the receptor. For example AChRs may arrive in the plasma membrane predominantly in a phosphorylated, active form, and then slowly become dephosphorylated, perhaps through the action of a membrane-associated phosphatase, to produce receptors that are less active. Rephosphorylation by a cAMP-dependent protein kinase might then convert the receptors back to the more active form. An alternative possibility is that AChRs appear in the membrane in an active form, and then slowly become associated with another component that restricts their function. The role of the cAMP-dependent process in this case might be to release AChRs from association with the restrictive component.

While the results are consistent with a single event being responsible for the changes, they do not exclude a host of other possibilities such as the existence of several competing control mechanisms whose balance or access changes with receptor age. The primary significance of the results presented here is that they demonstrate a cAMP-dependent modulation of the nicotinic response and indicate a "maturation" of AChRs in the plasma membrane with respect to function and regulation by secondary messenger systems. In view of the similarity between neuronal and adrenal chromaffin AChRs, it seems likely that similar conclusions may hold for at least some classes of neuronal receptors, implying that the regulatory mechanisms involved could influence the detection of synaptic input at neuronal as well as endocrine targets.

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